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Research Note

Cryopreservation of Infective Third-stage Larvae of *Strongyloides ratti*

THOMAS J. NOLAN AND GERHARD A. SCHAD

University of Pennsylvania School of Veterinary Medicine, Department of Pathobiology, 3800 Spruce St., Philadelphia, Pennsylvania 19104-6050

ABSTRACT: Infective third-stage larvae of *Strongyloides ratti* were successfully cryopreserved using a modification of the procedure developed for *Strongyloides stercoralis*. The larvae were frozen in a mixture of DMSO and dextran (10% of each in water) in the vapor phase of liquid nitrogen. Cryopreserved larvae were thawed into RPMI-1640 cell culture medium, and incubated overnight in an invertebrate saline to allow time for injured worms to die. The surviving larvae accounted for only 1% to 10% of those frozen, but when they were injected into rats a patent infection was produced.

KEY WORDS: *Strongyloides ratti*, cryopreservation.

Strongyloides ratti, a parasite of rats, is a frequently used laboratory model for species of *Strongyloides* that infect domestic animals and man. Because the rat eliminates an *S. ratti* infection in 3 to 4 wk, it is expensive to maintain this parasite, especially if more than 1 strain is being used. In fact, when we decided recently to re-establish this parasite in our laboratory, we were unable to find a laboratory in the United States that was maintaining it. Although both *Strongyloides stercoralis* (Nolan et al., 1988) and *Strongyloides papillosus* (Van Wyk et al., 1977) have been cryopreserved, the latter was not infective for its host (sheep) upon thawing. Therefore, this investigation was undertaken to determine whether our method for freezing *S. stercoralis* would not only cryopreserve *S. ratti*, but also maintain its infectivity.

The strain of *S. ratti* used in this investigation was G-60 (given to us by Dr. M. E. Viney, University of Edinburgh, Edinburgh, Scotland). This is a heterogonic strain originally isolated from a wild rat by Dr. G. Graham at the University of Pennsylvania. In our laboratory this strain was maintained in both Wistar rats and multimammate rats (*Mastomys natalensis*). Third-stage infective larvae (L_3) were obtained from 7-day-old coprocultures by baermannization, and washed twice in distilled water. These larvae were held in the freezing medium described for *S. stercoralis* (10% DMSO and 10% dextran; Nolan et al., 1988) for 15 to 90 min, depending on the experiment. They were then frozen in the vapor phase of liquid nitrogen and stored there for 7 to 330 days. The length of time spent frozen had no effect on the survival of the larvae, as was also described for other parasitic nematodes (Nolan et al., 1988; Van Wyk et al., 1990).

The larvae were thawed as described (Nolan et al., 1988) for *S. stercoralis* and were then resuspended in BU, a buffered saline designed for invertebrates (Hawdon and Schad, 1991). It is important to wash the larvae several times after thawing in order to remove all of the freezing medium since it is slightly toxic to the L_3's. Counts made 30 min after thawing showed no significant difference in survival (as judged by movement) between larvae frozen after incubation at room temperature for 15 to 90 min (Fig. 1). However, when the same larvae were counted approximately 20 hr after being thawed, survival had decreased significantly in all groups, but significantly more survived in the group given a 60-min incubation than in either the 15- or 30-min

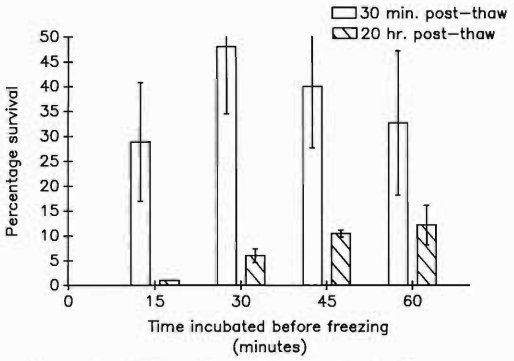

Figure 1. Effect of pre-freezing incubation in cryoprotectant on the survival of cryopreserved *Strongyloides ratti* third-stage larvae (L_3). Combined results of 8 experiments. Error bars = 1 standard deviation.

groups ($P < 0.05$, Mann-Whitney U-test). Using the same freezing method, the survival of *S. ratti* L_3's (32.7 ± 14.5%), as measured 30 min after thawing (60-min incubation group), was lower than that seen for *S. stercoralis* (55.0 ± 5.5%; Nolan et al., 1988).

When either multimammate or Wistar rats were injected subcutaneously with cryopreserved larvae within 1 hr of thawing, the infection became patent in very few animals (Table 1). However, when the larvae were incubated overnight in BU at 25°C, all rats that were injected developed patent infections (Table 1). After this overnight incubation, only about 1% of the thawed larvae were highly active, although 10.2% were still alive (a decrease from 32.7% at 30 min after thawing). Live larvae were separated from dead with a modified baermann apparatus. Tissue paper was placed in a sieve that was partially submerged in BU, the thawed larvae were placed on the tissue paper and incubated for 1 to 2 hr before they were collected from the BU by centrifugation. Only small numbers of larvae were finally recovered, suggesting that only the highly active larvae were penetrating the tissue paper.

Coprocultures made from Wistar rats injected with larvae 24 hr after thawing produced enough L_3's to infect another rat that developed a patent infection. From this rat, normal numbers of L_3's were obtained from charcoal-fecal cultures. Although rats receiving between 1,000 and 2,500 L_3's 24 hr after thawing developed patent infections, only a few adult worms were found in their intestines 10 to 14 days postinjection (Table 1). To increase the number of worms becoming adult, rats were immunosuppressed with methylprednisolone acetate (Depo-Medrol, Upjohn, Kalamazoo, Michigan). Subcutaneous injections of methylprednisolone acetate (2.5 mg/kg) were given on day −1, day 0, and days 5, 8, and 12. The 2 rats thus immunosuppressed were found to have more adult worms than the untreated rats (Table 1), but still far fewer than rats given third-stage larvae that had never been cryopreserved. When 1,000 normal L_3's were given to 2 rats, an average of 316 adult worms was recovered from the small intestine 10 days postinjection.

Although the system developed for freezing *S. stercoralis* (Nolan et al., 1988) is less effective for *S. ratti,* it can be used to cryopreserve the latter provided that the following modifications are

Table 1. Infections of rats with cryopreserved *Strongyloides ratti* third-stage larvae (L_3).

Dose of L_3's	Host	Time of injection of L_3's (after thawing)	Immuno-suppressed?	Result of charcoal-fecal culture	Prepatent period (days)	No. of worms recovered*
3,000	*Mastomys*	<1 hr	no	−	NA	ND
3,000	*Mastomys*	<1 hr	no	−	NA	ND
10,000	*Mastomys*	<1 hr	no	+	7	ND
12,000	*Mastomys*	<1 hr	no	−	NA	ND
20,000	*Mastomys*	<1 hr	no	−	NA	ND
1,000	*Rattus*	<1 hr	no	−	NA	0 (10)
1,000	*Rattus*	>20 hr	no	+	6	2 (10)
2,000	*Rattus*	>20 hr	no	+	7	0 (11)
2,500	*Rattus*	>20 hr	no	+	7	1 (14)
2,500	*Rattus*	>20 hr	yes	+	8	10 (14)
9,000	*Rattus*	>20 hr	yes	+	6	13 (27)

* (Day postinjection on which worms were recovered).
NA = not applicable; ND = not done.

used: 1. Because the recovery after thawing is less than 1%, large numbers of L_3's must be frozen (>100,000). 2. Upon thawing, the larvae should be incubated in BU at room temperature for 20 to 24 hr, allowing the injured L_3's to die. Thereafter, the living L_3's are collected. 3. Although it is not absolutely necessary, immunosuppression of the rat, before and after the subcutaneous injection of the L_3's, can be used to increase the number of adults maturing in the small intestine. 4. Because few adults develop from cryopreserved L_3's, it is necessary to amplify the infection by passage through another rat to obtain sufficient *S. ratti* for most experimental purposes.

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Research Note

Histochemical Observations on *Cyathostoma lari* (Strongyloidea: Syngamidae)

W. THRELFALL
Department of Biology, Memorial University, St. John's, Newfoundland, Canada A1B 3X9

ABSTRACT: The location and relative abundance of chemical components of adult female *Cyathostoma lari* were determined using a variety of histochemical methods. Carbohydrates, acid mucopolysaccharides, lipids, and proteins were widely distributed throughout the nematode. Host blood and hemoglobin were detected in the gut lumen, with hemoglobin also being demonstrated in the pseudocoel. Ribonucleic acid, mitochondria, succinic dehydrogenase, and acid and alkaline phosphatases were located in the digestive and reproductive systems. The data suggest that the anterior intestine plays the most important role in digestion. The role and importance of the various substances are discussed.

KEY WORDS: histochemistry, Nematoda, *Cyathostoma lari*.

Much work has been performed on the physiology and biochemistry of metazoan parasites, particularly digeneans and cestodes (von Brand, 1979; Chappell, 1980; Barrett, 1981; Smyth and McManus, 1989). One of the least examined groups has been the nematodes, with species of medical and veterinary importance receiving the most attention. Within this group larvae and eggs have been the objects of greatest attention. In examining the chemical constituents of nematodes, studies have to a large extent been performed using homogenates of whole worms (Singh and Sharma, 1981; Chopra, 1986; Rao and Rajlingam, 1989). Few studies have been concerned with determining the precise location of various substances within nematodes (Sood and Kalra, 1977; Sood and Sehajpal, 1978; Maki and Yanagisawa, 1980; Sharma and Singh, 1985).

The distribution of the chemical components of a parasite is a reflection of where biochemical processes are occurring, with intensity and type of reaction perhaps changing at different times during the host's life cycle. Host physiology and location of the parasite within the host will also affect the physiological status of the parasite. Phylogenetic differences among hosts and among parasite species might also be reflected in the parasite's physiology. As noted by Threlfall et al.